Developmental stage specificity of the lymphoid *V(D)J* recombination activity

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We have examined the level of immunoglobulin gene *V(D)J* recombination activity in a number of cell lines derived from lymphoid or nonlymphoid lineages. The assay we employed uses extrachromosomal DNA as substrate and thereby avoids difficulties associated with the use of chromosomally integrated substrates. The recombination activity decreases during B-lymphoid development. It is highest at the earliest stages of committed B-cell differentiation and then falls progressively, reaching undetectable levels at the mature B-cell stage. The activity is also present in multipotential progenitors of myeloid cells and in pre-T cells but not mature T cells. No activity was found in several nonhematopoietic cell lines. Recombination was seen only among substrate molecules which had replicated in the eukaryotic cells. Several possible interpretations of this result are discussed.

[*Key Words:* Recombination; immunoglobulin; T-cell receptor; lymphoid development]

Received July 1, 1987; revised version accepted August 4, 1987.

The diversity in antigen recognition by immunoglobulins (Ig) and T-cell receptors (TCR) is due primarily to site-specific recombination of genomic DNA sequences called *V* (variable), *D* (diversity), and *J* (joining) segments to form the mature genes encoding these proteins. Recombination is directed by joining signals, sequences in the noncoding DNA adjacent to each of these coding segments. Each joining signal sequence consists of a highly conserved palindromic heptamer whose consensus sequence is CACAGTG and an A/T-rich nonamer (reviewed by Alt et al. 1986; Kronenberg et al. 1986).

The overall temporal order of immunoglobulin rearrangement has been inferred from studies on transformed lymphoid cell lines (for review, see Yancopoulos and Alt 1986). Heavy-chain *D_H* and *J_H* segments join on both chromosomes, followed by *V_H*-to-*D_HJ_H* joining. A productive *VD_H* joining, though it does not necessarily shut off further rearrangement at the heavy-chain locus (Kleinfield et al. 1986), appears to signal the onset of light-chain gene rearrangement (Reth et al. 1985).

In previous studies of this gene rearrangement reaction, rearrangements of either endogenous or exogenous but chromosomally integrated segments were monitored (Blackwell and Alt 1984; Lewis et al. 1984, 1985). From these studies two factors appear important: the presence of the recombination activity and the accessibility of the recombination substrate. A previous study (Yancopoulos et al. 1986) of an integrated exogenous substrate has suggested that eligibility for recombination correlates with transcription of the surrounding DNA.

To assess the activity of the recombination enzyme system in various cell lines without effects due to the

chromosomal context of the substrate DNA, we have employed an assay that utilizes an extrachromosomal circular DNA substrate containing the joining signal sequences (Hesse et al. 1987). Because the assay can be shown to detect recombination only among extrachromosomal DNA molecules that have replicated, it is possible to measure recombination frequency rather accurately and to disregard DNA that is merely absorbed to the cells.

We report a survey of *V(D)J* recombination activity present in transformed cells that represent various tissues and various developmental stages, with particular attention to the successive stages of B- and T-cell development. The results strongly suggest that the *V(D)J* recombination activity is regulated during B- and T-cell differentiation in a stage-specific manner.

Results

The strategy used here to measure levels of recombination activity employs a plasmid, pJH200 (Fig. 1), which contains the heptamer–nonamer joining signals as well as genes conferring antibiotic resistance. This substrate DNA is transfected into the eukaryotic cells to be tested, recovered 48 hr later, and introduced into *E. coli* by transformation. All plasmid DNA confers ampicillin resistance (Amp^r). Because recombination excises a transcription stop signal upstream of the chloramphenicol acetyl transferase (CAT) gene in pJH200, recombinant molecules also confer chloramphenicol resistance (Cam^r). Thus, the ratio of doubly resistant (Amp^rCam^r) colonies to Amp^r colonies reflects the fraction of DNA that is rearranged at the heptamer–nonamer joining signals. *Hgi*AI restriction analysis of plasmids recovered

Figure 1. Outline of recombination reaction. Plasmid pJH200 contains two heptamer–nonamer immunoglobulin joining-signal sequences flanking a prokaryotic transcription terminator (labeled “stop”). The signals, in turn, are flanked by the *E. coli* *plac* promoter on one side and the gene for CAT on the other. The terminator prevents expression of the *cat* gene when the plasmid is in *E. coli*. After *V(D)J* recombination in lymphoid cells (lower part of figure), the terminator has been deleted, allowing *cat* expression in *E. coli*, and the joining signals have been fused, heptamer to heptamer. The signal joint contains a novel *HgiAI* restriction site. Plasmid pJH200 is identical to pJH201, previously described (Hesse et al. 1987), except that pJH200 lacks the κ transcription enhancer.

from doubly resistant colonies (Hesse et al. 1987) confirmed that a precise signal junction (heptamer-to-heptamer fusion) was formed in the large majority of recombinants. The exceptional cases will be the subject of a separate publication.

The quantitative interpretation of the experimental data is complicated by the variable fraction of exogenously added DNA that can remain stuck to the outside of the eukaryotic cells. This can lead to spuriously low estimates of the frequency of recombination. Therefore, we took advantage of the fact that plasmid pJH200 can replicate autonomously in murine cells because it contains the early region of the polyoma genome. Unreplicated molecules, which retain a prokaryotic methylation pattern, can be eliminated by digestion of the recovered DNA with the restriction enzyme *DpnI* prior to transformation of the bacteria. Because we find *V(D)J* recombination only among replicated DNA molecules (as described below), digestion with *DpnI* preferentially removes DNA molecules that have had no opportunity to recombine. Because it gives a more reliable measure of recombination, the ratio of Amp^rCam^r to Amp^r among replicated molecules, which we call *R*, is our preferred way of presenting recombination frequencies.

V(D)J recombination activity in the B-lymphoid lineage

To assess the variation in *V(D)J* recombination activity during B-lymphoid differentiation, we measured the activity in a number of transformed cell lines that are

thought to represent B cells at various stages of development. The cell lines used are listed in Table 1, grouped by lineage and developmental stage. The progenitor B- and pre-B-cell lines are further classified according to the stage of rearrangement of their Ig genes (heavy chain, κ or λ light chain) and according to the designations given in the original descriptions of the cell lines.

All 18 cell lines representing pre-B or earlier stages contain *V(D)J* joining activity, except for the relatively mature 70Z/3 cell line. Among these lines, the recombination activity varies in a systematic way. The least differentiated cell lines, BAMC1 and HAFTL1, have detectable but quite low recombination activity. These cells have been designated pro-granulocyte-monocyte-B lymphocyte (pro-GMB), because subclones can follow either the monocytic or lymphoid pathway (Holmes et al. 1986). Thus, they represent a developmental stage before commitment to the B-lymphoid lineage has occurred.

The cell lines at the next stage (pro-B), BASC6 and NFS70, have much higher recombination activity. In fact, these cells had the highest levels of recombination among those we tested, with 43% and 100% of replicated molecules being rearranged, respectively. These cells express some but not all surface markers characteristic of pre-B cells (Davidson et al. 1984; Holmes et al. 1986; Mushinski et al. 1987); on the other hand, their subclones have not been observed to differentiate along any other than the B-cell pathway. They thus represent very early committed B cells.

At later stages of B-cell development, recombination activity generally appears to decrease; however, we observed large variations from one cell line to another. Among seven pre-B cell lines at the stage of heavy-chain rearrangement, there is a 20-fold range of activity (from 1.3% to 27%). These cell lines include some derived from fetal liver and others derived from 2- to 3-week-old or adult murine bone marrow; there is no obvious correlation of recombination activity with the original source of the cells. Two of these lines, 38B7 and 1-8, are of particular interest. Both heavy-chain alleles of 38B7 have undergone nonproductive VD_H rearrangement and have exhausted their D segments. In the 1-8 line, there has been a nonproductive VD_H rearrangement at one heavy-chain allele and a DJ_H rearrangement at the other. Neither line shows continuing rearrangement of its endogenous light- or heavy-chain Ig genes (Alt et al. 1984), even though our assay detects an active recombination system in both lines. Therefore, the failure of the endogenous genes to rearrange appears to reflect the state of those loci rather than the absence of activity.

Among five cell lines that are active at rearranging the κ locus (Lewis 1985), the recombination activity is still lower. Three of these lines make cytoplasmic μ -chains whereas two do not, but this property is not correlated with their recombination activity. A similar lack of correlation is seen among subclones of the κ -rearranging cell line PD, some of which make cytoplasmic μ -chains whereas others do not (see below; Table 2).

The ABC-1 cell line, which is active in rearranging both the κ and λ loci (Lewis 1985; Persiani et al. 1987) has considerably higher activity than those that rearrange only κ .

Among three cell lines at the late pre-B- or mature B-cell stage, two were recombination negative and one was positive. The 70Z/3 cell line, which was negative, is classified as pre-B because it is not surface Ig positive, though it does make cytoplasmic μ and a low level of cytoplasmic κ chains (Wall et al. 1986). This relatively mature pre-B line has never been demonstrated to rearrange its already productively rearranged endogenous Ig heavy and κ loci any further; it did not have detectable activity in our assay. The mature B-cell line, WEHI-279 (Harris 1978), which is surface Ig $\mu^+\kappa^+$, also failed to show any recombination activity. In contrast, the mature B-cell line BALB 1427 (Mushinski et al. 1987) did have recombination activity, although 100% of the cell population is surface Ig $\mu^+\kappa^+$. Thus, expression of surface Ig does not by itself seem to be sufficient to shut off V(D)J recombination activity.

Four cell lines representing the latest stages of B-cell differentiation were all recombination negative (Table 1). All of these bear, in addition to surface Ig, high levels of the surface marker PC-1, a characteristic of the plasma cell stage. At this stage, the recombination activity appears to be conclusively absent.

The recombination-positive cell lines described above were derived by neoplastic transformation with a number of different viruses (see Table 1 legend). There was no obvious correlation between recombination activity and the method used to obtain the cell line.

V(D)J recombination activity in pre-T and early myeloid cells

Two pre-T cell lines (Risser et al. 1985) are recombination positive. One of these, 2052C, has been shown to rearrange its γ and β loci in culture, whereas the other, 2017, is already rearranged at γ and β (R. Spolski, R. Risser, and T. Mak, pers. comm.). Since the mature T-cell line B6Mo3 (Risser et al. 1978) has no recombination activity in this assay, the V(D)J recombination activity may also be developmentally regulated in the T-cell lineage.

The M1 cell line (Ichikawa 1969) had recombination activity in our assay (Table 1). These cells lack several markers characteristic of monocytic differentiation, but gain Mac-1, Mac-3, lysozyme production, and phagocytic capability upon stimulation with lipopolysaccharide (Ralph et al. 1983). They can thus be induced to differentiate along the monocyte-macrophage pathway. M1 also retains the ability to be induced to differentiate along the granulocytic pathway (Tsuda et al. 1986); however, it has never been observed to differentiate along the lymphoid pathway. Detection of V(D)J recombination activity in this line may indicate persistence of the activity early after the divergence of myelo-monocytic cells from the lymphoid pathway. Two terminally differentiated myeloid cell lines, RAW309 Cr.1 and P815, were recombination negative. The erythroid precursor Friend erythroleukemia line F-MEL was negative for V(D)J recombination activity.

Absence of V(D)J recombination in other tissues

None of a variety of cell lines representing nonhematopoietic lineages showed detectable recombination. The cells tested include fibroblast, neuroblastoma, smooth muscle, embryonic hepatocyte, and epithelial cell lines (Table 1).

Variation in recombination among subclones

Do different subclones of the same transformed cell line vary markedly in recombination activity? Some variation among subclones of the PD cell line was found; the overall range was about fivefold (Table 2). These subclones vary in the length of time they have been cultured since viral transformation, in the status of their κ alleles, and in μ -chain synthesis (Lewis 1985), but there was no apparent correlation of recombination activity with any of these parameters. Moderate variations in recombination were also found in subclones of the 18-8 (Clark and Rosenberg 1980) and ABC-1 cell lines (Table 2). These differences have to be calibrated against the variability in repeated assays of the same cell line, which is typically twofold from top to bottom of the range.

Recombination is detected only among replicated DNA molecules

Plasmid pJH200 and the closely related plasmid pJH201 (Hesse et al. 1987), which differs in containing the κ enhancer region, are capable of replicating autonomously

Table 1. Developmental stage and tissue specificity of the V(D)J recombination activity

Tissue and stage description	Literature designation of transformant	Cell line name	Genotypic and phenotypic parameters							Rep Amp ^r	Rep Amp ^r Cam ^r	R × 100	References
			Ig Loci			μ protein	Ly5 (B220)	Thy-1	Amp ^r				
			H	κ	λ								
Nonhematopoietic													
Neural cell	Neuroblastoma	Neuro-2A							114,000	100,000	0	<.001	1
Embryonic liver	—	BNL CL.2							4,000	4,400	0	<.02	1
Epithelial cell	Carcinoma	CMT-93							800	630	0	<.2	1
Fibroblast	—	L Cell							31,000	31,000	0	<.003	
Fibroblast	—	Psi 2							5,600		0	<.02	2(3)
Smooth muscle	—	BC ₃ H1							400	240	0	<.4	1
Hematopoietic													
B-lymphoid lineage													
Progenitor B and Pre-B (stage of heavy-chain locus rearrangement)	Pro-GMB	BAMC1	R	G	—	—	—	3,000	680	1	0.2	4(5)	
	Pro-GMB	HAFTL1	G,R	G	—	(+)	—	15,000	600	6	1.0	4(5)	
	Pro-B	BASC6 C2	R	G	—	—	—	15,000	24	24	100	4(5)	
	Pro-B	NFS 70	R		—	(+)	—	19,000	7	3	43	1(6)	
	Pre-B	HS1C5	R	G	—	(+)	—	1,100	150	6	4.0	4(5)	
	Pre-B	BAC14	R	G	—	(+)	—	600	31	6	19	4(5)	
	Pre-B	38B7	R	G	—			7,500	1,800	85	4.7	7(8)	
	Pre-B	1-8	R	G	—	+	—	1,500	860	149	17	9(8,10)	
	Pre-B	22D6	R	G	—	+		3,500	930	255	27	9(8,10)	
	Pre-B	38B9	R	G	—	+		1,200	250	14	5.7	9(8,10)	
	Pre-B	40E4	R	G	—			14,000	1,800	24	1.3	9(8)	
	Pre-B (stage of kappa locus rearrangement)	Pre-B	18-81	R	R	+	+	—	6,500	970	8	0.8	7(8,10)
		Pre-B	PD31	R	R	—	+	—	13,000	6,150	28	0.5	11(10,12)
		Pre-B	196-313	R	R	+			43,000	2,100	31	1.5	11(13)
		Pre-B	143-2M3	R	R	—			7,800	8,100	358	4.4	7(13)
		Pre-B	204-1-7	R	R	+			12,400	3,300	52	1.6	7(8,13)
	Pre-B	70Z/3	R	R	+			2,000	630	0	<0.2	1(8)	
	Pre-B (stage of lambda locus rearrangement)	Pre-B	ABC-1	R	R	R	+		19,000	67	13	19	11(13)
Mature B cell	B Cell	BALB-1427	R	R	+	+		30,000	560	8	1.4	15(14)	
	B Cell	WEHI-279	R	R	+			175,000	880	0	<0.1	1	
Immunoblast	Immunoblast	SJL-4	R	R	+	+		4,100	1,750	0	<0.06	15(14)	
Plasma cell	Plasmacytoma	S194/5.XXO-1	R		+	+		2,500	1,360	0	<0.07	1	
	Plasmacytoma	MOPC460D	R	R	+	+		4,300	2,100	0	<0.05	16(17)	
	Plasmacytoma	MPC 11	R	R	+			15,000	1,100	0	<0.09	11(18)	
T-lymphoid lineage													
Pre-T			TCR loci										
			γ	β	α								
	Pre-T	2052C	G	G	G		—	8,700	240	2	0.8	19(20)	
	Pre-T	2017	R	R	R		+	32,000	6,700	17	0.3	19(20)	
Mature T	T Cell	B6Mo3					+	24,000	5,100	0	<0.02	19	
Monocytic lineage													
Monoblast	Myeloblast	M1						110,000	1,900	5	0.3	1(21)	
Monocyte	Monocyte	RAW309						11,000	1,800	0	<0.06	1	
Granulocytic lineage													
Mast cell	Mastocytoma	P815						77,000	1,600	0	<0.06	1	
Erythroid lineage													
Early precursor	Early precursor	F-MEL						4,300	1,200	0	<0.08	22	

Table 2. Comparison of V(D)J recombination activity in clonally related cell lines

Subclone or subline	Approximate days in culture since viral transformation	IgH	κ	λ	μ -Chain expression	Amp ^r	Rep Amp ^r	Rep Amp ^r Cam ^r	R $\times$ 100
PD	58	RR		GG	+	13,000	2,050	44	2.1
PD 31	76	RR	GG	GG	–	14,000	8,600	68	0.8
PD 31	190	RR		GG	–	13,000	6,150	28	0.5
PD 34-4	124	RR	RG	GG	+	5,800	4,500	85	1.9
PD 34-14	124	RR	DR	GG	+	2,700	2,250	10	0.4
PD 34-28	118	RR	DD	GG	+	11,000	2,000	7	0.4
ABC 1		RR	DD	RR	+	19,000	67	13	19
AT 2		RR	DD	RR	+	16,000	1,500	297	20
18-81		RR	RR	GG	+	6,500	970	8	0.8
18-82		RR	RR	GG	+	8,200	1,950	47	2.4
18-84		RR	RR	GG	+	1,100	130	2	1.5

Subclones were transfected with pJH200; plasmid DNA was recovered 48 hr later and analyzed using the *E. coli* transformation assay (see Methods). R is the fraction of replicated substrate which underwent V(D)J recombination. PD and its subclones and ABC-1 and its subline (AT-2) have been characterized by Lewis (1985) for endogenous Ig locus status, μ -chain expression, and time in culture after viral transformation. Subclones of 18-8 (18-81, 18-82, and 18-84) were characterized and described by Clark and Rosenberg (1980). (RR) Rearranged at both alleles; (RG) rearranged at one allele and germ line at the other; (DR) deleted at one allele and rearranged at the other; (GG) germ line at both alleles.

in mouse cells because they contain the early region of the polyoma virus genome. The following series of experiments was carried out with pJH201. To assess the fraction of transfected molecules that had replicated, plasmid DNA recovered from the cells was digested with the restriction endonucleases *DpnI* or *MboI*. These enzymes both cleave at the sequence GATC, but have complementary specificities in that *DpnI* cuts only if the A in GATC is methylated on both strands whereas *MboI* cuts only if both As are unmethylated. Neither enzyme cuts at hemimethylated sites. The transfecting plasmid DNA was grown in *dam*⁺ strains of *E. coli* and thus was initially methylated at these sites. Because methylation of As is not propagated during replication in eukaryotic cells, molecules that have undergone one or more rounds of replication in these cells are hemimethylated or unmethylated and are resistant to *DpnI*. The sequence GATC occurs over 20 times in pJH200 and pJH201, so that sensitive molecules will be exten-

sively fragmented and unable to give rise to transformants after introduction into bacterial hosts.

In the experiments summarized in Table 3, cells were transfected with pJH201, and the DNA was recovered and digested with *DpnI* or *MboI* or with both enzymes. A fraction of each digestion mix was transformed into *E. coli* and Amp^r or Amp^rCam^r transformants were scored.

The fraction of *DpnI*-resistant DNA molecules varied from 1% at 24 hr to as much as 66% at later times (Table 3). For each cell line, the *MboI*-resistant fraction represented nearly all of the remainder; the sum of Amp^r transformants obtained with DNA that was digested with *DpnI* or *MboI* approximately equalled that obtained with undigested DNA. This result, which suggests that few hemimethylated molecules were recovered, was confirmed by double digestion with *DpnI* and *MboI* (Table 3).

It is to be noted that recombined molecules (Amp^rCam^r) are detected in the replicated pool but not in the

Table 1. (continued)

Cell lines were transfected with pJH200, and plasmid was recovered 48 hr later. The recovered plasmid was analyzed using the *E. coli* transformation assay. R is the fraction of replicated plasmid that underwent V(D)J recombination. The prefix Rep, as in RepAmp^r, designates the number of *DpnI*-resistant plasmids. Results are summations of 2–4 transfections per cell line. Staining for Ly5(B220) that is positive but lower than for normal cells is designated (+) (Davidson et al. 1984; Holmes et al. 1986). Abbreviations used in referring to Ig and TCR endogenous gene status: (G) germ line; (R) rearranged; (G,R) retention of germ line configuration at one allele and rearrangement at the other. The source of each cell line is given by the number in the references column. References for the phenotypic and genotypic characterization of each cell line are given by the numbers in parentheses. The numbers are keyed to the following: (1) American Type Culture Collection (ATCC) (originators of each cell line from ATCC are given in the ATCC volume *Cell Lines and Hybridomas*, 5th edition); (2) Richard Mulligan, MIT; (3) Mann et al. 1983; (4) Jaclyn Pierce, NIH; Hoelzer and Aaronson 1982; Pierce and Aaronson 1985; (5) Holmes et al. 1986; (6) Davidson et al. 1984; (7) Naomi Rosenberg, Tufts; Baltimore et al. 1979; (8) Alt et al. 1984; (9) David Baltimore, MIT; (10) McKearn and Rosenberg 1985; (11) Susanna Lewis, NIH; (12) Lewis et al. 1982; (13) Lewis 1985; (14) Mushinski et al. 1987; (15) Kevin Holmes and Herbert Morse, NIH; (16) Rick Norden and Michael Potter, NIH; (17) Jaffe et al. 1969; (18) Laskov and Scharff 1970; (19) Rex Risser, U. Wisconsin; (20) R. Spolski, R. Risser, and T. Mak (pers. comm.); (21) Ralph et al. 1983; (22) Arthur Nienhuis, NIH. The method of growth transformation of the hematopoietic cells is given in the original description cited for each line. Abelson murine leukemia virus was used in all but the following cases: HAFTL-1 and HS1C5, Harvey sarcoma virus; BAMC1 and BASC 6, BALB sarcoma virus; NFS-70, Cas-NS-7; BAC 14, 2017 and B6Mo3, Moloney murine leukemia virus; 70Z/3, methyl nitrosourea; BALB 1427 and SJL-4, retroviral constructions (Rapp et al. 1986); S194/5.XXO-1 and MOPC460D, mineral oil; MPC 11, solid plastic-induced; M1, spontaneous leukemia; P815, DBA/2 mouse tumor; and F-MEL, Friend virus.

Table 3. *Recombination among replicated DNA molecules*

Cell line	Time after transfection (hr)	Amp ^r				Percent DNA replicated	Amp ^r Cam ^r				Percent undigested DNA recombined	R × 100
		Rep	Nonrep	Hemimethylated	Undigested		Rep	Nonrep	Hemimethylated	Undigested		
22D6	48	900	2,400	0	3,200	28	87	0	0	80	3	10
1-8	24	900	78,000	0	80,000	1	34	0	0	36	0.05	4
	36	2600	2,500	2	4,700	55	209	0	0	200	4	8
	48	1600	840	0	2,400	67	183	0	0	169	7	11

22D6 or 1-8 cells were transfected with pJH201, and plasmid DNA was recovered at the indicated times. An aliquot of each recovered plasmid was digested with *DpnI* or with *MboI*. In each experiment, equivalent amounts of undigested and digested DNA were transformed into *E. coli*. (Rep) Number of colonies resulting from transformation of *E. coli* with *DpnI*-digested DNA. (Nonrep) Number of colonies resulting from transformation with *MboI*-digested DNA.

unreplicated pool. Some molecules undoubtedly fail to replicate and recombine because they are merely adsorbed to the cells. There may be additional reasons why replication is required for recombination (see Discussion), but as a practical matter it is reasonable to exclude from the calculation of recombination frequency those DNA molecules that are ineligible for recombination. Thus, the fraction of recombined among replicated molecules (R) is a better measure of recombination proficiency than the overall recombination frequency. In cases where no recombination occurs, replication documents that the DNA did enter the nucleus.

The results above show that accessibility of DNA to replication and recombination enzymes goes in parallel. Could DNA undergo *V(D)J* recombination if it fails to replicate for reasons other than its location? We addressed this question by constructing a plasmid (pJH205) identical to pJH201 but having a linker insertion mutation at the polyoma origin (Kern et al. 1986) which markedly depresses initiation of DNA replication.

As expected, plasmid pJH205 does indeed replicate to a much smaller extent than pJH201 in 1-8 cells. After 40 hr, replication of pJH205 was only 2% of that of pJH201 (Table 4). Nevertheless, R was similar for both plasmids, as is also shown in Table 4.

As a further test of the need for replication, we constructed a plasmid (pJH100) that is generally similar to pJH201 except that it lacks the polyoma early region altogether. At 40 hr after transfection into 1-8 cells, there was no detectable replication of pJH100, and no recombination. Parallel transfections of pRSVcat (Gorman et al. 1982) resulted in CAT expression, documenting that DNA entered the lymphoid cells. No Amp^rCam^r colonies were recovered from a transformation with an amount of DNA corresponding to 2×10^5 Amp^r transformants, whereas a parallel transfection with pJH201 resulted in 4000 Amp^rCam^r colonies among 50,000 replicated molecules.

Time course of replication and rearrangement in pre-B cell lines

Replication of the plasmid was first seen at 18 hr after transfection, and the fraction of recovered molecules

that had replicated increased for several hours thereafter (Fig. 2A). The peak for maximal recovery of replicated plasmid was between 35 and 45 hr. The time course of the absolute number of replicated molecules (not shown) was similar to that of polyoma DNA after infection of cultured cells (Dulbecco et al. 1965). Cotransfection of rearranged and unrearranged molecules into cells has shown that there is no detectable difference in the replication or degradation of these two forms of the plasmid (data not shown).

Recombination was typically detectable at 24 hr after transfection and increased for some time. R increased roughly linearly out to 60 hr here (Fig. 2B) and out to 72–84 hr in other experiments. This time course has been repeatable with separate cultures of the cell line 1-8 over a 1-year period.

To test the effect of the κ enhancer, we transfected 1-8 cells with pJH201, which contains the enhancer, and pJH200, which does not. There was no difference in the time course of recombination or replication (data not shown). The same was true when both plasmids were tested in PD31. Furthermore, R values determined at 48 hr were essentially the same for both substrates in each cell line of Table 1. It should be noted that both plasmids already contain the polyoma enhancer adjacent to the replication origin.

Discussion

Our results show that *V(D)J* recombination is confined to cell lines from the lymphoid and myeloid lineages. Cells derived from other hematopoietic lineages (Fig. 3)

Table 4. *Replication and recombination in plasmids with variant replication origins*

	Amp ^r	RepAmp ^r	RepAmp ^r Cam ^r	R × 100
pJH201	21,800	22,500	977	4
pJH205	4,550	91	3	3
pJH100	200,000	0	0	—

pJH201, pJH205, or pJH100 was transfected into 1-8 cells, plasmid DNA was recovered at 40 hr and analyzed for replication and recombination using *DpnI* and the *E. coli* transformation assay. The prefix Rep, as in RepAmp^r, designates the number of *DpnI*-resistant plasmids.

Figure 2. Time course of replication and recombination. 1-8 cells were transfected with pJH201 as described in Methods, but on a 10-fold larger scale. Samples were aliquoted immediately after transfection; one was taken at each indicated time, and plasmid DNA was recovered. (A) Percentage of DNA that has been replicated (scored as Amp^r colonies in DNA resistant to *DpnI* digestion per total Amp^r colonies) or recombined (scored as Amp^rCam^r colonies in DNA resistant to *DpnI* digestion per total Amp^r colonies). (B) Ratio R of recombined to replicated DNA is presented as a function of time.

Figure 3. Distribution of *V(D)J* recombination activity among hematopoietic cell types. The pathway of hematopoietic differentiation is summarized. Known lineage relationships are indicated by solid lines, more tenuous relationships by dashed lines. Specific cell lines representing each stage are named below it. A circled (+) or (-) above the cell stage indicates the presence or absence of detectable *V(D)J* recombination activity in the corresponding cell lines. See text and Table 1 for details.

Figure 4. Recombination frequency in cell lines of the B lineage. R values taken from Table 1 are plotted as a function of the developmental stage of the cell lines; each point represents one cell line. The boxes enclose the range of R in that category of cells. For each stage, the current or most recent locus of Ig recombination is indicated: (H) heavy-chain locus; (λ) λ locus; (κ) κ locus.

and from nonhematopoietic lineages (Table 1) did not rearrange the extrachromosomal substrates, at least at the level of sensitivity used here (generally below 0.1%, except in cell lines where poor replication or low transfection efficiency render the assay less sensitive). Within the B-lymphoid lineage (Fig. 4), recombination is low at the earliest stage tested (pro-GMB), higher in pro-B cells, but lower in cell lines representing successive later stages. The λ-rearranging line ABC-1 falls outside this apparent progression, if one accepts the conventional view that λ chain rearrangement follows unsuccessful κ rearrangement (see, e.g., Hieter et al. 1981; Tonegawa 1983). However, more recent data suggest that some λ-rearranging cells may form a distinct B-lymphoid sub-lineage (Hardy et al. 1986), in which case models based on a single sequence of rearrangements would need to be modified. One mature B-cell line that is surface Ig positive retains the activity, implying that the presence of tetrameric surface Ig is not by itself sufficient to shut off $V(D)J$ recombination activity. Activity is undetectable in immunoblast and plasma cell lines. Recombination is

also seen in pre-T cell lines but not in a mature T cell line.

Prior to this study, $V(D)J$ recombination activity had not been directly demonstrated in cell lines capable of differentiation along both the myeloid and the B-lymphoid pathways. The finding of occasional D/H rearrangements in monocytic leukemias (Bauer et al. 1986) and the occurrence of lymphoid blast crises in monocytic leukemias (Bakhshi et al. 1983) have suggested that the recombination activity might be present in a common precursor. Our finding of activity in such cells is in accord with these observations. The persistence of $V(D)J$ recombination in the myelo-monocytic precursor line M1, which is not known to differentiate along the lymphoid lineage, indicates that the activity appears to persist even beyond the divergence of myeloid precursors from the lymphoid pathway.

How is recombination activity distributed among the cells in a population? When a large fraction of the plasmid DNA is recombined, the majority of transfected cells evidently must participate. However, when the activity is low, as it is at the very early and very late stages of B-cell differentiation, we do not know whether it is equally low in all cells or whether a small fraction of cells accounts for most of the recombination.

Earlier work has suggested that $V(D)J$ recombination is regulated, at least in part, at the level of substrate accessibility (Yancopoulos and Alt 1986). The ordering of endogenous rearrangements (TCR or Ig, joining of D to J before V to DJ , IgH before κ or λ) is best rationalized by assuming that there is a common recombinase, which is granted access to a locus only at certain developmental stages. Our data are consistent with this suggestion, because the same extrachromosomal substrate DNA can be recombined in many cell lines, regardless of the stage of rearrangement that the endogenous genes are undergoing. Indeed, we find high recombination activity in some cell lines whose endogenous gene rearrangement has been arrested. Recombination of the same substrate in pre-T cells and in monocytic precursors as well as pre-B cells is most easily understood if a common recombination activity is present in all these cell types.

The role of replication

We detect $V(D)J$ recombination only among those DNA molecules that have been replicated in the eukaryotic cells. No recombinants have been found that are resistant to digestion by *MboI* and are thus unreplicated. The reason why replication is necessary to detect a signal in our system is still unclear. In a plasmid with a defective polyoma origin (pJH205), replication and recombination are depressed in parallel (Table 4). However, there is no necessary correlation between the rates of recombination and replication. In some cell lines, such as BASC6, where replication of the plasmid DNA is poor, there are high levels of recombination; the opposite situation is also found, as in PD31 cells. Furthermore, once some replication has occurred, additional replication can be inhibited with aphidocolin or by shifting to a nonpermissive temperature when a plasmid with a *ts* mutation

in the polyoma large T antigen is used, without blocking later recombination (unpubl.). However, it is not certain that replication was entirely blocked in these two situations.

Why should replication, either prior or concurrent, be needed to observe recombination in our assay? A simple possibility is that multiplication of the input DNA or the recombined product is needed to give a large enough signal to be scored. However, other explanations are also conceivable. At one extreme, one could imagine that replication is an active part of the recombination process; for instance, the passage of a replication fork could be a necessary triggering event. On the other hand, it is possible that prior replication is only needed to convert the DNA to a state where recombination is allowed, by altering its methylation pattern, or state of chromatin coverage or some other persistent property.

We have not specifically sought to examine the association of transcription and recombination. Our replication-competent substrates should be transcriptionally active (Chou et al. 1974; Kern et al. 1986). However, several variants of pJH200 in which polyoma replication is fully blocked but transcriptional controls are unaltered fail to recombine at a measurable level. Therefore, transcription without replication does not permit detectable recombination in our assay. Further, we have performed experiments using α -amanitin to block RNA polymerase II transcription, and the drug had no effect on further rearrangement of pJH200 when added after the onset of recombination (data not shown).

Methods

Plasmid constructions

The V(D)J recombination substrate pJH201 has been described (Hesse et al. 1987). Plasmid pJH200 was constructed identically to pJH201, except that the Ig κ enhancer was not included.

The construct pJH100 is similar to pJH201, except that the *trp* promoter (purchased from P-L Pharmacia) was positioned in the place of the *lac* promoter and the early region of polyoma is absent.

The plasmid pJH205 is similar to pJH200, except that the early region of polyoma comes from p4819 (a gift from S. Lipton, A. Cowie, and R. Kamen), which has an *Xho*I linker inserted in the polyoma origin of replication between polyoma bases 36 and 38 (Soeda numbering).

Osmotic transfection of cells

Based upon an osmotic transfection procedure that we had previously devised for erythroid cells (Hesse et al. 1986; Lieber et al. 1987), we varied the osmotic conditions during DEAE-dextran transfection of lymphocytes. The osmotic rupture of the lymphocytes was monitored by following lactate dehydrogenase (LDH) release. CAT expression and recombination substrate replication during the 48 hr after transfection were optimal when the LDH release in the hypotonic DEAE-dextran-DNA transfer step and the subsequent media wash step added up to 60% of the total LDH content of the transfected cell population. These transfection conditions are described below.

Cells were cultured as described previously (Hesse et al. 1987). Cells (3×10^6) at a suspension density of approximately 1×10^6 to 2×10^6 /ml were pelleted (440 g for 5 min), washed

once with 0.5 ml 100% RPMI 1640, repelleted, and resuspended in 0.5 ml of DEAE-dextran-DNA [67% (vol/vol) RPMI 1640, 0.05 M Tris HCl, pH 7.45, 250 μ g/ml of DEAE-dextran (P-L Pharmacia Biochemicals, $\sim 5 \times 10^5$ daltons), and 0.3 μ g/ml of DNA], which was prepared a few minutes before use. This effectively hypotonic solution exposes the cells to a controlled osmotic stress, which we have found to enhance DNA uptake. Alternately, 100 mM NH_4OAc , a membrane-permeable salt, was substituted for 67% RPMI 1640. The reaction mixture was incubated 5–30 min (depending on the time of optimal LDH release) at 37°C, after which the cells were pelleted, washed once with 0.5 ml 100% RPMI 1640, repelleted, and resuspended in 0.5 ml RPMI/serum/antibiotics. After the pH was adjusted with CO_2 , the transfected cells were incubated at 37°C in 1.5-ml Sarstedt tubes tightly capped and lying on their sides. With this method, 50% of 1–8 cells are transfected, as inferred from positive immunostaining for polyoma T antigens (not shown).

Nonhematopoietic cells were adherent and were transfected in 150-cm² culture flasks. They were washed twice with Dulbecco's PBS at 22°C and then incubated for 4–8 hr at 37°C in 5 ml of the DEAE-dextran-DNA solution described above for suspension cells. The cells were washed once in Dulbecco's PBS at 22°C and incubated in medium at 37°C for designated times, typically 48 hr.

Recovery of the plasmid DNA from transfected cells and transformation into *E. coli* were as described previously (Hesse et al. 1987).

Digestion with *Dpn*I or *Mbo*I was carried out under the conditions described by the manufacturer (New England Biolabs). A reaction volume of 5 μ l, containing 2 U of enzyme and 0.02–2 ng of recovered plasmid DNA, was incubated for 6–14 hr at 37°C. The fraction of unreplicated DNA able to transform *E. coli* after *Dpn*I digestion was less than 10^{-5} .

Acknowledgments

We thank S. Lewis, A. Cowie, A. Szabo, R. Martin, J. De Villiers, and R. Deans for valuable discussions. We are indebted to A. Cowie, M. Israel, and J. Bolan for polyoma DNA and antibodies to T antigens and to R. Risser, S. Lewis, N. Rosenberg, J. Pierce, F. Alt, D. Baltimore, K. Holmes, H. Morse, W. Davidson, R. Norden, and M. Potter for cell lines.

We thank S. Lewis, R. Craigie, R. Martin, and G. Selzer for critical reading of the manuscript. M.L. acknowledges the support of the Laboratory of Pathology, NCI, National Institutes of Health and the Council for Tobacco Research (Grant 1867) during portions of this work.

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Developmental stage specificity of the lymphoid V(D)J recombination activity.

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Genes Dev. 1987, 1:

Access the most recent version at doi:[10.1101/gad.1.8.751](https://doi.org/10.1101/gad.1.8.751)

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